Combining Azadirachtin and *Paecilomyces fumosoroseus* (Deuteromycotina: Hyphomycetes) to Control *Bemisia argentifolii* (Homoptera: Aleyrodidae)

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ABSTRACT Both azadirachtin and *Paecilomyces fumosoroseus* (Wize) Brown & Smith have been used to control the whitefly *Bemisia argentifolii* Bellows & Perring, but with only moderate effectiveness. Azadirachtin is a botanical insecticide derived from the neem tree, and *P. fumosoroseus* is an entomopathogenic fungus. To test whether these two agents might be more effective for whitefly control if used together, different rates of each were combined in laboratory bioassays in factorial treatment. Both tank mixes and separate sprays were tested. Up to 90% nymphal mortality was obtained when both the fungus and azadirachtin were combined, a significant increase over the 70%, or less, mortality obtained when only one agent was used; however, the combined effects were less than additive. Azadirachtin had moderately inhibitory effects on growth and germination of *P. fumosoroseus*, which may explain this antagonism.

KEY WORDS azadirachtin, *Bemisia*, *Paecilomyces*, microbial control

Bemisia tabaci (Gennadius) is a complex of whitefly species with uncertain taxonomy (Perring 2001). What has been known as the 'B' biotype is probably a separate species, *B. argentifolii* (Bellows & Perring), that is extremely polyphagous and a serious crop pest worldwide, especially in warm climates and greenhouses (Perring 2001). Pathogen outbreaks can severely limit *B. tabaci* and *B. argentifolii* populations, and nearly all pathogens described from these whiteflies are hyphomycete fungi, with most naturally occurring outbreaks caused by *Paecilomyces, Verticillium,* or *Aschersonia* spp. (Lacey et al. 1996). *P. fumosoroseus* has been developed for inundative release as a spray application for control of *B. argentifolii*, and such applications can be effective in the field (Wraight et al. 2000), as well as in the greenhouse (Vidal et al. 1998). At least one *P. fumosoroseus* product is registered with the Environmental Protection Agency as a microbial pesticide, but it is not currently produced in the U.S. As with other microbial pesticides of whiteflies, *P. fumosoroseus* has its limitations, including slow action (several days), sensitivity to weather conditions, poor persistence, and high cost of production (Faria and Wraight 2001).

Azadirachtin, a steroid-like tetranortriterpenoid derived from neem trees (*Azadirachta indica* Juss.), is a strong antifeedant and growth regulator for a wide variety of phytophagous insects, including whiteflies. It delays and prevents molting, reduces growth, development and egg production; and can cause significant mortality in whitefly immatures (Coudriet et al. 1985, Flint and Parks 1989, Prabhaker et al. 1989, Schmutterer 1990, Liu and Stansley 1995). It has been tested in the field with variable success (Puri et al. 1994, Leskovar and Boales 1996, Akey and Henneberry 1999), and is commercially available in the U.S. for whitefly control.

Whiteflies are notoriously difficult to control because they are multivoltine, highly fecund, and often have high immigration rates. In addition, they are hard to control with spray applications because they feed on the abaxial surface of leaves, where they are difficult to reach. Chemical control has further been complicated by *Bemisia*'s ability to quickly develop resistance to chemical pesticides (Prabhaker et al. 1985). The purpose of the experiments reported here were to test whether microbial control with *P. fumosoroseus* could be improved with the use of azadirachtin.

Materials and Methods

Source of the Fungus. *P. fumosoroseus* 612, was obtained from the USDA-ARS laboratory in Peoria, IL (from Mark Jackson) where it is being used to develop fermentation and formulation methods for microbial control of whiteflies. We used this culture to infect silverleaf whitefly nymphs in the laboratory, and then

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reisolated it as a pure culture from infected nymphs. After reisolation, this strain was always grown on Saboraud dextrose agar (Difco Laboratories, Detroit, MI) modified with 1% yeast extract and 1% chloramphenicol (SDAY) for 14 d at 25°C with a photoperiod of 10:14 (L:D) h. Petri plates were sealed with parafilm for 1 week, after which time the parafilm was removed to allow the plates to dry out and to stimulate the production of conidial spores. For any given experiment, the conidia used to inoculate the culture plates were never >4 culture cycles past an original reisolate from whitefly nymphs. Freshly collected conidia from 14-d-old cultures were used for every experiment and each replicate run.

Effects of Azadirachtin on Spore Germination. Azadirachtin (Neemix 4.5, Certis, Columbia, MD) was added to SDAY at the following rates: 0, 30, 60, and 90 μg azadirachtin/ml medium, and then poured into 100 by 15 mm diameter petri plates. *P. fumosoroseus* conidia were mixed in a sterile, aqueous solution of 0.01% Silwet L-77 (Loveland Industries, Greeley, CO), and the concentration was adjusted to 1×10^8 conidia/ml using a hemocytometer. Each plate was inoculated with 0.1 ml of spore suspension that was spread evenly over the plate. Each treatment had four replicate plates that were incubated at 25°C in the dark.

After 20 h of incubation, the proportion of spores that had germinated was determined by observing the spores at 400× magnification with a phase contrast microscope. The sample size was 300 spores per replicate plate. Analysis of variance (ANOVA) and Tukey's student *t*-test (SAS Institute 2001) was used to test whether any of the treatments differed significantly from each other. An arcsine-square-root transformation was used to normalize the data.

Effects of Azadirachtin on Spore Production. Using the same concentrations of azadirachtin in SDAY that were tested above, 1×10^7 *P. fumosoroseus* conidia were spread onto four 100 × 15 mm plates of each treatment, and then incubated at 25°C, with a photoperiod of 14:10 (L:D) h, for 14 d. The plates were sealed with parafilm for the first 7 d of this incubation, then the parafilm was then removed and the plates were incubated at 70% RH in a growth chamber, using the same temperature and photoperiod. The number of spores produced per plate was determined by flooding each plate with 15 ml of sterile, aqueous 0.01% Silwet. The spores were mixed into suspension by carefully rubbing a sterile, bent, stainless steel rod over the culture surface, and then drawing off a subsample of the spore suspension (10 ml) to determine the spore concentration using a hemocytometer (Goettel and Inglis 1997), and then the number of spores per plate was estimated. ANOVA, followed by Tukey's student *t*-test was used to test whether spore production differed significantly between any of the treatments.

Source of *B. argentifolii* Nymphs. *B. argentifolii* nymphs were maintained on cantaloupe, eggplant, and hibiscus plants in a walk-in growth chamber at ≈25°C, with a photoperiod of 14:10 (L:D) h. Adults were allowed free-flight in the chamber. For each experiment, sweet potato leaves were collected from plants grown in a greenhouse, and then soaked in a weak solution of bleach and dish detergent in cold water (exact proportions were not measured) for several minutes to remove any mites, whiteflies, fungi, and other pests that might have been on the leaves. The leaves were then rinsed three times in cold water, and placed in water piks with nutrient solution (0.03% Peters Excel, The Scotts Co., Marysville, OH) and allowed to sit overnight to dry and regain turgor. The sweet potato leaves were able to develop roots and remain in good condition for several weeks in this manner.

These leaves were then placed in a screened cage that was inside the walk-in whitefly chamber described above. Whitefly adults from the colony were shaken from their host plants into the cage until enough whiteflies were present to infest plants with at least 40 eggs per leaf (based on previous experience) when left 48 h. After 48 h, the leaves were removed from the cage and all the adult whiteflies were removed by blowing them off (by mouth). The leaves were incubated in another growth chamber at 25°C with a photoperiod of 14:10 (L:D) h, for 10 d. After incubation, each leaf was examined under a dissecting scope and 40 nymphs in the early second instar were marked by placing a small black dot next to each, using a fine tipped permanent marker. The mark was placed away from the nymph so that the ink did not affect the insect. If the insects were so crowded together in a particular spot that it was not easy to identify which one was supposed to be marked, then a very fine insect pin was used to remove some of the other nymphs. Any leaves with <40 nymphs in the second instar were discarded. The nymphs and leaves were treated the day after being marked.

Spray Application Method. Leaves were sprayed using a Potter Precision Laboratory Spray Tower (Burkhard Manufacturing, Rickmansworth, England) with 0.7 kg/cm^2 and the fine-mist nozzle (as described by James and Jaronski 2000). Each leaf was laid out flat on an acrylic plate with the abaxial surface facing up (and the rooted stem remaining in the water pik), and then sprayed with 1 ml of each treatment preparation (treatments are described below). After being sprayed, all the leaves were set upright by placing the water piks in test tube racks such that the leaves did not touch or overlap each other. The leaves were allowed to air dry in the laboratory, and then each rack of leaves was covered with a plastic bag to raise the humidity to ≥95%. The leaves were not arranged in any particular order within each rack. All the racks were incubated at 25°C with a photoperiod of 14:10 (L:D) h, and a small temperature and relative humidity recorder (Hobo H8 ProSeries, Onset Computer Corp., Pocasset, MA) was placed in one of the bags. The leaves were removed from the bags after 24 h and incubated at 70% RH, and the same temperature and photoperiod. Insect mortality was recorded 7 d after each spray application.

Determining Treatment Application Rates. To determine fungal spore delivery rate, in every experimental run a block of 15% agar ($\approx 20 \times 20 \times 3$ mm) was sprayed along with a *P. fumosoroseus*-treated leaf. The density of spores on the block was counted under a microscope at 400×. For each agar block, the number of spores in 0.1, 0.4, and 2.6 mm^2 was counted for the high, medium and low application rates, respectively. These areas yielded counts of 150–500 spores. On the first replicate of the first experiment, only three agar blocks were counted for each fungus application rate. For all subsequent replicates and experiments, an agar block was placed next to every *P. fumosoroseus*-treated leaf, thus eight blocks were used to estimate spore density (see treatments described below). All the blocks within a treatment were pooled, and the standard errors reported later are based on the variance among replicates.

To estimate the amount of azadirachtin delivered, the fungal spore densities were used to estimate the volume of water applied per mm^2 of leaf surface. This number was multiplied by the known concentration of azadirachtin in a delivered solution to estimate the concentration of azadirachtin per mm^2 of leaf surface.

Spore viability was tested at the time of each experimental run by spraying a plate of SDAY with 1 ml of 1×10^8 conidia/ml (with no azadirachtin). The plate was incubated for 20 h in the same incubator as the treated insects. Percent germination was determined using a sample of 300 spores, as described above. Spore germination rates were always ≥99%.

Testing for Interactive Effects Against the Whitefly. The effect of combining azadirachtin and *P. fumosoroseus* on survival of silverleaf whitefly nymphs was tested using 4 ×4 factorial. Treatments were four concentrations of azadirachtin (0, 15, 30, and 60 μg/ml), four of *P. fumosoroseus* spores (0, 5×10^7, 1×10^8, and 2×10^8 conidia/ml), and all possible paired combinations of each control agent. The recommended field rate for azadirachtin (based on the label) is 12–48 g (AI)/ha. If one were to use 285 liters/ha (30 gal/acre), these rates would yield a tank concentration of 42–168 μg/ml. Thus, the azadirachtin rates we tested were probably at the moderate to low end of the recommended application rates, although it is always difficult to convert between field and laboratory rates. The concentrations were selected for the whitefly bioassays because azadirachtin started to show some toxic effects against *P. fumosoroseus* at 90 μg/ml (see results below). Furthermore, by selecting field application rates in the moderate to low end, we enhanced our ability to detect interactive effects with the fungus and to determine what the lowest effective dose might be when combined with the fungus. *P. fumosoroseus* was not commercially available, so field application rates for our strain was uncertain, thus we selected a range of application rates that we expected to yield between 20 and 90% mortality, based on previous experiments in our laboratory, and on published literature (Wraight et al. 1998).

Three experiments were conducted using different methods for combining azadirachtin and *P. fumosoroseus:* (experiment 1) a tank mix of azadirachtin and conidia was sprayed onto whitefly infested leaves, (experiment 2) azadirachtin was sprayed onto infested leaves 2 h before *P. fumosoroseus* was sprayed, and (experiment 3) azadirachtin was sprayed onto infested leaves 3 d before *P. fumosoroseus.* For experiment 2, the plants sat in the laboratory under ambient conditions (≈25°C) for the 2 h between the applications. For experiment 3, plants were placed in an incubator at 25°C, 70% RH, 14:10 (L:D) h for the 3 d between treatments.

Table 1. Effect of azadirachtin on conidial germination and production of *P. fumosoroseus* conidia

Azadirachtin Concentration (μg/ml)	% Spore Germination (SE)	Number of spores produced per plate $\times 10^9$ ($\pm$ SE)
0	100 (0.0) a	14.9 (1.3) a
30	99.5 (0.2) ab	10.3 (0.7) b
60	99.1 (0.2) b	5.4 (1.4) c
90	69.1 (1.1) c	1.7 (0.4) c

Means for germination are significantly different ($F = 360$, df = 3, $P \leq 0.05$) if they are not followed by the same letter. Means for spore production also are significantly different ($F = 29.7$, df = 3, $P \leq 0.05$) if they are not followed by the same letter.

Each experiment was replicated three times, and each replicate conducted on a different date. For each replicate run, two leaves were used per treatment for a total of 80 insects per replicate. The tank mix experiment was done first, and the replicates for the second two experiments were alternated over the following 6 weeks.

Within a given experiment, all the treatments had the same control and thus it was not necessary to adjust mortality based on Abbott's adjustment, which is intended to compensate for differential background mortality (such as differences in the affects between different formulations when trying to compare insecticide effects [Abbott 1925]). A two-way ANOVA was used to determine whether azadirachtin and *P. fumosoroseus* had significant effects on nymphal mortality, and whether the interaction between the two was significant. For each replicate in each experiment, all the treatments were set up at one time using the same stock solutions of azadirachtin and spores, thus the experiment was a randomized block design and time was included in the ANOVA as the block effect. An arcsine-square-root transformation was used on insect mortality to normalize the data.

Results

Direct Effects of Azadirachtin on the Fungus. Some negative effects of azadirachtin were seen when *P. fumosoroseus* conidia were plated onto SDAY containing azadirachtin. Spore germination was 100% in the absence of azadirachtin, but decreased when the compound was present (Table 1). Although germination at 60 μg/ml of azadirachtin was significantly lower than the control, the difference was extremely small (100% versus 99.1%). The effect of azadirachtin on germina-

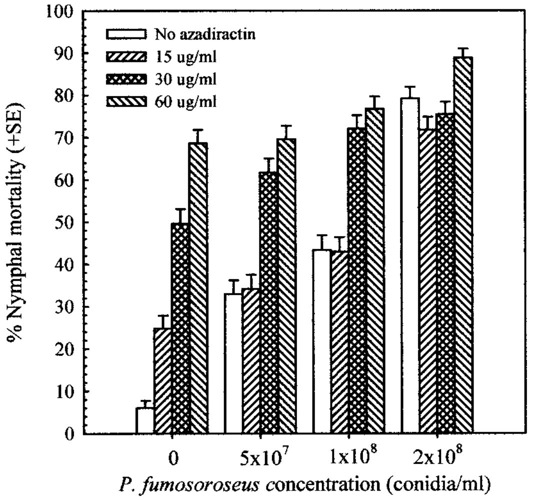

Fig. 1. Mean mortality of second instar whiteflies when sprayed with a tank mix of azadirachtin and *Paecilomyces fumosoroseus* conidia at different rates and combinations. Mortality was measured 7 d after treatment. Lines at the top of each bar represent the standard errors of the means.

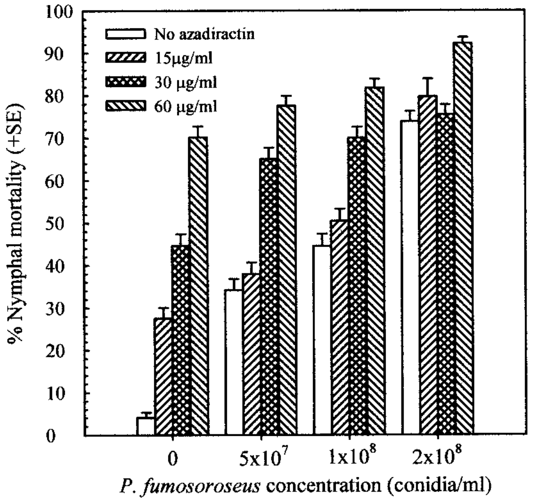

Fig. 2. Mortality of second instar whiteflies when azadirachtin was applied 2 h before *Paecilomyces fumosoroseus* conidia were applied. Mortality was measured 7 d after treatment. Lines at the top of each bar represent the standard errors of the means.

tion was more pronounced at 90 μg/ml. Spore production was also reduced significantly in the presence of azadirachtin (Table 1). At 30 μg/ml, only 70% as many spores were produced as in the control, even though germination of the original inoculum was reduced by only 0.5%. At 90 μg/ml, the number of spores produced was only ≈12% of that in the control.

Application Rates in the Whitefly Bioassays. In the tank mix experiment, the fungus was applied at rates of 107 (SE = 24), 536 (83), and 1383 (123) conidia/mm^2 for the spray concentrations of 5×10^7, 1×10^8, and 2×10^8 conidia/ml, respectively. For the experiment where azadirachtin and *P. fumosoroseus* were sprayed separately but 2 h apart, the spore densities were 119 (7), 476 (52), and 1739 (351) spores/mm^2, respectively. For the experiment where the sprays were applied three days apart, spore densities were 192 (6), 717 (52), and 1879 (34) spores/mm^2, respectively. The estimate of the delivery volume for each spray varied with spore concentration, but when averaged over all spray applications, it was estimated to be 5.6×10^{-6} (SE = 9.2×10^{-5}) ml solution/mm^2. The corresponding rates of azadirachtin applied would then be ≈0.336, 0.168, and 0.084 ng/mm^2 for the 15, 30, and 60 μg/ml concentrations, respectively.

Tank Mix Experiment. Azadirachtin and *P. fumosoroseus* both significantly increased insect mortality ($F = 114$; df = 3, 32; $P \leq 0.0001$ and $F = 125$; df = 3, 32; $P \leq 0.0001$, respectively). Combining these two control methods did not have an additive effect on mortality (the interaction term in the ANOVA was significant, $F = 12$; df = 9, 32; $P \leq 0.0001$). The fact that the two control measures, when applied together, were less than the sum of each applied alone (Fig. 1) is an indication that the two compounds are antagonistic. For example, in the absence of *P. fumosoroseus*, mortality increased by ≈20% between the rates of 30 and 60 μg/ml of azadirachtin. Similarly, in the absence of azadirachtin, mortality increased by ≈40% between the medium and high rates of *P. fumosoroseus.* However, the medium rate of *P. fumosoroseus* combined with the 30 μg/ml rate of azadirachtin yielded ≈60% mortality, and when either *P. fumosoroseus* or azadirachtin were increased to the next higher rate, the mortality rate increased by <10%. The degree of antagonism was not so great as to make combining the two control agents counter productive; the highest mortality still occurred when both azadirachtin and *P. fumosoroseus* were combined.

Separate Sprays, 2 h Apart. Again, both azadirachtin and *P. fumosoroseus* had significant effects on mortality ($F = 207$; df = 3, 32; $P \leq 0.0001$ and $F = 213$; df = 3, 32; $P \leq 0.0001$; respectively), and the interaction between the two was statistically significant ($F = 16$; df = 9, 32; $P \leq 0.0001$) (Fig. 2). The results were very similar to what was seen with the tank mix. Azadirachtin and *P. fumosoroseus* each yielded similar mortality rates when they were applied independently at the medium and high concentrations. Mixing the two again yielded the highest mortality rates, but the effect was still less than additive.

Sprays Separated by 3 d. In this experiment, combining azadirachtin and *P. fumosoroseus* resulted in a more linear response (Fig. 3). The interaction between the two was significant 7 d after azadirachtin was sprayed ($F = 3$; df = 9, 32; $P \leq 0.01$), but not after 10 d ($F = 2$; df = 9, 32; $P \leq 0.09$), at which time it had been 7 d since *P. fumosoroseus* had been applied. As expected from the previous experiments, the effects of azadirachtin and *P. fumosoroseus* were significant at day 7 ($F = 80$; df = 3, 32; $P \leq 0.0001$ and $F = 99$; df = 3, 32; $P \leq 0.0001$, respectively), and again on day 10

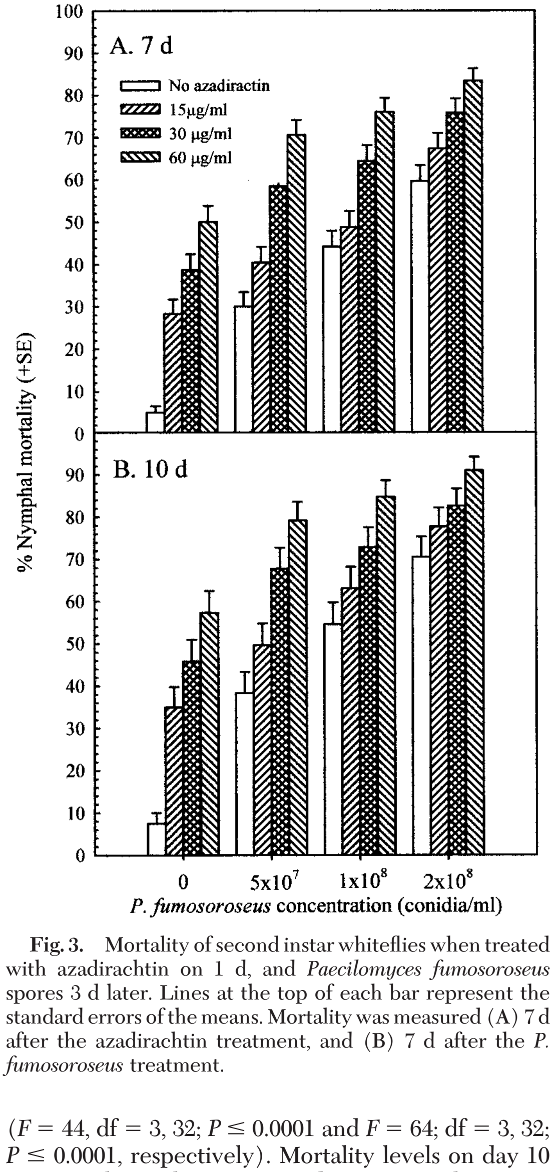

Fig. 3. Mortality of second instar whiteflies when treated with azadirachtin on 1 d, and *Paecilomyces fumosoroseus* spores 3 d later. Lines at the top of each bar represent the standard errors of the means. Mortality was measured (A) 7 d after the azadirachtin treatment, and (B) 7 d after the *P. fumosoroseus* treatment.

($F = 44$, df = 3, 32; $P \leq 0.0001$ and $F = 64$; df = 3, 32; $P \leq 0.0001$, respectively). Mortality levels on day 10 were similar to those seen on day seven in the experiment where azadirachtin and *P. fumosoroseus* treatments were separated by only 2 h.

Discussion

Spore germination was affected by concentrations of azadirachtin at or above 60 μg/ml, but spore production was more sensitive. Reductions in the number of spores produced when azadirachtin was present could have been because of inhibitory effects on either fungal growth rate or conidial formation. A reduced rate of germination in the inoculum could also explain some of the inhibitory effects, but the reduction in spore germination was only a fraction of the reduction seen in spore production, so it was not enough to entirely explain the result. The antifungal activity was not strong enough to completely inhibit the fungus; in fact, it was difficult to visually distinguish differences in fungal growth among culture plates, except for the difference between the 90 μg/ml and the other treatments.

Although combining azadirachtin and *P. fumosoroseus* increased insect mortality, the effects were not additive or synergistic, but were inhibitory. The degree of inhibition was small enough that, in most cases, it did not eliminate the benefits of combining the two compounds. Synergy is a preferable situation, of course, because it allows growers to apply less material. Azadirachtin acts as an insect growth inhibitor, and the purpose behind delaying the application of *P. fumosoroseus* was to allow sufficient time for azadirachtin have an effect on insect metabolism, and then determine whether such effects would induce the insects to be more susceptible to fungal infection. Unfortunately, no such effect was found. However, delaying the *P. fumosoroseus* application did reduce the antagonistic effects that azadirachtin had on the fungus.

James and Elzen (2001) found an inhibition between *Beauveria bassiana* (Balsamo) Vuillemin and imidacloprid when applied to silverleaf whitefly nymphs, yet the chemical did not have any apparent fungicidal effects on the pathogen. In that case, the effects were likely a result of changes in insect behavior induced by the fungus that reduced the uptake of imidacloprid. However, such an effect is not likely to explain the results seen in this study because the main mode of action of azadirachtin is through contact toxicity.

It is much more likely that the antagonism was because of a direct, toxic effect of azadirachtin on *P. fumosoroseus*. Azadirachtin was used in the whitefly bioassays at rates that had minimal effects on *P. fumosoroseus* germination and growth, in vitro, but it is possible that any effects on the fungus were compounded in vivo, before or during the time infection was initiated. Previous studies have found crude extracts from neem to have anti-fungal activity. For example, neem extract can be effective in preventing powdery mildew on roses (Pasini et al. 1997) and peas (Singh and Prithiviraj 1997), and various plant pathogenic fungi in chickpeas (Singh et al. 1980). Conversely, neem extracts inhibit aflatoxin biosynthesis in *Aspergillus flavus* Link ex. Fries, but do not inhibit the fungus from colonizing cotton seeds (Zeringue and Bhatnagar 1990). In all of these studies, azadirachtin was undoubtedly in the extracts, but it is not clear whether it was the component responsible for the anti-fungal activity. However, such activity would be consistent with the results of this study.

In summary, azadirachtin and *P. fumosoroseus* can increase whitefly mortality when combined, resulting in mortality levels that are greater than either product yielded on its own, but the effect is less than additive. The greatest mortality of silverleaf whitefly nymphs was observed when the highest rate of azadirachtin was followed by the highest rate of *P. fumosoroseus*. Because of the high production costs of azadirachtin and *P. fumosoroseus*, and the lack of a synergistic effect between these two biological control strategies, combining the two agents probably does not provide an economically feasible solution to whitefly control problems.

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